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Attorney Docket No. 5721-5

First Named Inventor or Application Identifier: Mary S. Cupp,
et al.

Express Mail Label No. EM166859923US

A/BOX SEA

ADDRESS TO: ASSISTANT COMMISSIONER FOR PATENTS
BOX PATENT APPLICATION
WASHINGTON, DC 20231

Transmitted herewith for filing in the United States Patent Office is a patent application for:

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Title of Invention: ANTITHROMBIN PROTEIN AND DNA SEQUENCES

1. The Filing Fee has been calculated as shown below:

(Submit an original, and a duplicate for fee processing)

No. Filed	No. Extra	Small Entity Rate	Large Entity Rate
BASIC FEE		\$ 395	\$ 790
TOTAL CLAIMS:	17 - 20 =	x 11 = \$ 0	x 22 = \$
INDEP CLAIMS:	4 - 3 =	x 41 = \$ 41	x 82 = \$
[] MULTIPLE DEPENDENT CLAIMS PRESENTED		+135 = \$	+270 = \$
*If the difference in Column 1 is less than zero, enter "0" in Column 2.		TOTAL \$ 436	TOTAL \$

The Commissioner is hereby authorized to credit overpayments or charge the following fees to Deposit Account No. 16-0605.

- a. Fees required under 37 CFR 1.16 (National filing fees).
- b. Fees required under 37 CFR 1.17 (National application processing fees).
- A check in the amount of \$436 is enclosed.

2. Specification; Total Pages 22

3. Sheets of Formal Drawing(s) (35 USC 113)

4. Declaration and Power of Attorney; *[Total Pages 1]*
a. Newly executed (original or copy)
b. Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed) [Note Box 5 below]
i. DELETION OF INVENTOR(S) Signed statement attached deleting
inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) & 1.33(b).

5. Incorporation By Reference *(usable if Box 4b is checked)*. The entire disclosure of the prior
application, from which a copy of the oath or declaration is supplied under Box 4b, is considered
as being part of the disclosure of the accompanying application and is hereby incorporated by
reference therein.

6. Microfiche Computer Program (Appendix)

7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
a. Computer Readable Copy
b. Paper Copy (identical to computer copy)
c. Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. Assignment Papers (cover sheet & document(s) (including \$40.00 fee)

9. 37 CFR 3.73(b) Statement *(when there is an assignee)*; Power of Attorney

10. English Translation Document *(if applicable)*

11. Information Disclosure Statement (IDS)/PTO-1449; Copies of IDS Citations

12. Preliminary Amendment

13. Return Receipt Postcard (MPEP 503) *(Should be specifically itemized)*

14. Small Entity Statement(s)
 Statement filed in prior application; status still proper and desired.

15. Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
Foreign Priority is

16. Other: _____

17. If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:
 Continuation Divisional Continuation in Part (CIP)

**This application claims the benefit of U.S. Provisional Application No: 60/040,683; filed March
13, 1997.**

18. CORRESPONDENCE ADDRESS

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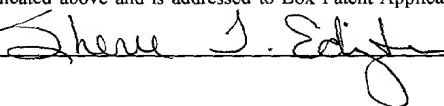
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Attorney Docket No. 5721-5

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Cupp *et al.*

Serial No.: To be determined

Filed: March 6, 1998

For: ANTITHERMOBIN PROTEIN AND DNA SEQUENCES

March 6, 1998

STATEMENT IN SUPPORT OF FILING A
SEQUENCE LISTING UNDER 37 CFR § 1.821(f)

BOX SEQUENCE

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I hereby state that the content of the paper and computer readable copies of the Sequence listing, submitted concurrently herewith in accordance with 37 CFR § 1.821(c) and (e), are the same.

Respectfully submitted,



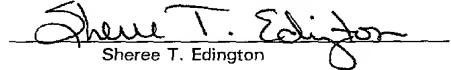
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Sheree T. Edington

ANTITHROMBIN PROTEIN AND DNA SEQUENCES

CROSS-REFERENCE

This application claims the benefit of U.S. Provisional Application No. 60/040,683, filed March 13, 1997.

FIELD OF THE INVENTION

The present invention relates to medical treatments utilizing antithrombin proteins.

BACKGROUND OF THE INVENTION

Thromboembolic diseases are among the most important circulatory diseases. A thrombin is a blood clot that partially or completely blocks blood flow through a blood vessel. An embolus is a thrombus that has formed elsewhere in the body, broken free, and traveled to the site where blockage occurs. Blockage in the brain results in a stroke, i.e., a cerebral infarction, a localized area of dead cells. An embolus in a lung can produce pulmonary embolism, one of the principal lung diseases in bed-ridden patients. Bed ridden and elderly persons are also particularly prone to thrombophlebitis, which is a blockage of circulation in a leg caused by an embolus. An embolus or thrombus lodging in one of the blood vessels serving the heart causes necrosis of part of the heart tissue, a myocardial infarction, commonly called a heart attack.

The initiating event of many myocardial infarctions is the hemorrhage into atherosclerotic plaques. Such hemorrhage often results in the formation of a thrombus (or blood clot) in the coronary artery which supplies the infarct zone. This thrombus is composed of a combination of fibrin and blood platelets. The formation of a fibrin-platelet clot has serious clinical ramifications. The degree and duration of the occlusion caused by the fibrin-platelet clot determines the mass of the infarct zone and the extent of damage.

The formation of fibrin-platelet clots in other parts of the circulatory system may be partially prevented through the use of anticoagulants, such as heparin. Unfortunately, heparin has not been found to be universally effective in preventing reocclusion in myocardial infarction victims in which the degree of blood vessel occlusion is greater than or equal to 70%, particularly in those patients with severe residual coronary stenosis. Among the more promising of the agents are hirudin and its analogs, which bind to and inactivate thrombin. Hirudin has a theoretical advantage over heparin as an anti-thrombotic agent. Thrombin bound to thrombi or platelets is relatively protected from inhibition by heparin while hirudin, at least *in vitro*, is still effective. Other promising investigational agents include fibrinogen receptor antagonists, which block platelet aggregation and dense granule release by a mechanism distinct from that of aspirin, and inhibitors of thromboxane production.

There is therefore a need for additional antithrombin agents which exhibit low toxicity, little or no antigenicity, and a very short clearance time from circulation.

25 SUMMARY OF THE INVENTION

Antithrombin proteins and DNA sequencing coding the proteins are provided. The protein named simulidin is isolated from *Simulium Spp.*. The proteins are useful in medical treatments where antithrombin agents are needed.

30 Methods for preparation and administration of the proteins are additionally provided.

DETAILED DESCRIPTION OF THE INVENTION

Methods and compositions for use as antithrombin agents are provided. The agents have an inhibitory effect on thrombin. The proteins are from black flies (Simuliidae) and other hematophagous Diptera belonging to the sub order Nematocera, particularly from *Simulium* ssp. The protein has been designated simulidin. A major function of the proteins of the invention is 1) to delay hemostasis by the abrogation of clotting by eliminating the formation of a fibrin network through initiation of the α -form of thrombin, and 2) inhibition of platelet aggregation stimulated by thrombin.

The compositions of the invention comprise antithrombin proteins from the salivary gland of the blood-feeding black fly. The proteins exhibit antithrombin activity as well as the ability to interfere with macrophage function. Substantially purified preparations of the protein are provided. Such substantially purified preparations include proteins substantially free of any compound normally associated with the protein in its natural state. Such proteins can be assessed for purity by SDS-PAGE, chromatography, electrophoresis or other methods. See, M.P. Deutscher (ed.), *Guide to Protein Purification*, Academic Press, Inc. (1990).

The terms "substantially pure" or "substantially purified" are not meant to exclude artificial or synthetic mixtures of the protein with other compounds. It is recognized that the antithrombin proteins of the present invention include those proteins homologous to, and having essentially the same biological properties as, the antithrombin protein described herein, and particularly the protein disclosed herein in SEQ ID NO: 2. This definition is intended to encompass natural allelic variations in the genes.

The invention additionally encompasses the nucleotide sequences which encode the proteins of the invention. The nucleotide sequence of the coding sequence from *S. vittatum* is provided in SEQ ID NO: 1. Additionally, cloned genes of the present invention can be of other species of origin. Such species include, but are not limited to *S. argus*, *S. ochraceum*, and *S. metallicum*.

DNAs which hybridize to the nucleotide sequence of the antithrombin gene from the black fly are also an aspect of this invention. Conditions, which will

permit other DNAs to hybridize to the DNA disclosed herein, can be determined in accordance with known techniques. For example, hybridization of such sequences may be carried out under conditions of reduced stringency, medium stringency or even stringent conditions (e.g., conditions represented by a wash stringency of 35-40% Formamide with 5x Denhardt's solution, 0.5% SDS and 1x SSPE at 37° C; conditions represented by a wash stringency of 40-45% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 42° C; and conditions represented by a wash stringency of 50% Formamide with 5x Denhardt's solution, 0.5% SS and 1x SSPE at 42°C, respectively, to DNA encoding the genes disclosed herein in a standard hybridization assay. See J. Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory)).

In general, sequences which code for the antithrombin protein and hybridize to the nucleotide sequence disclosed herein will be at least 75% homologous, 85% homologous, and even 95% homologous or more with the sequences. Further, the amino acid sequences of the antithrombin proteins isolated by hybridization to the DNA's disclosed herein are also an aspect of this invention. The degeneracy of the genetic code, which allows different nucleic acid sequences to code for the same protein or peptide, is well known in the literature. See, e.g., U.S. Pat. No. 4,757,006.

The hybridization probes may be cDNA fragments or oligonucleotides, and may be labeled with a detectable group as known in the art. Pairs of probes which will serve as PCR primers for the antithrombin gene or a protein thereof may be used in accordance with the process described in U.S. Patent Nos. 4,683,202 and 4,683,195.

The polypeptides of the invention may be subject to one or more post-translational modifications such as sulphation, COOH-amidation, acylation or chemical alteration of the polypeptide chain.

It is recognized that the nucleotide and peptide sequences of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the peptides and proteins can be prepared

by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel, T. (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; US Patent No. 4,873,192; Walker and Gaastra (eds.) *Techniques in Molecular Biology*, MacMillan Publishing Company, NY (1983) and the references cited therein.

5 Thus, the nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the peptides and proteins of the invention encompass both naturally occurring and modified forms thereof. Such variants will continue to possess the desired activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create sequences deleterious to expression of the gene product. See, EP Patent Application Publication No. 75,444.

10 Thus proteins of the invention include the naturally occurring forms as well as variants thereof. These variants will be substantially homologous and functionally equivalent to the native protein. A variant of a native protein is "substantially homologous" to the native protein when at least about 80%, more preferably at least about 90%, and most preferably at least about 95% of its amino acid sequence is identical to the amino acid sequence of the native protein. A variant may differ by as few as 1, 2, 3, or 4 amino acids. By "functionally equivalent" is intended that the sequence of the variant defines a chain that produces a protein having substantially the same biological activity as the native protein of interest. Such functionally equivalent variants that comprise substantial sequence variations are also encompassed by the invention. Thus a functionally equivalent variant of the native protein will have a sufficient biological activity to be therapeutically useful. By "therapeutically useful" 15 is intended effective in achieving a therapeutic goal as discussed below.

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25 Methods are available in the art for determining functional equivalence. Biological activity can be measured using assays specifically designed for measuring activity of the native protein, including assays described in the present invention. Additionally, antibodies raised against the biologically active native protein can be tested for their ability to bind to the functionally equivalent variant, where effective

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binding is indicative of a protein having conformation similar to that of the native protein.

DNA sequences can also be synthesized chemically or modified by site-directed mutagenesis to reflect the codon preference of the host cell and increase the expression efficiency.

The proteins of the invention can be "engineered" in accordance with the present invention by chemical methods or molecular biology techniques. Molecular biology methods are most convenient since proteins can be engineered by manipulating the DNA sequences encoding them. Genomic DNA, cDNA, synthetic DNA, and any combination thereof may be used for this purpose. Genomic DNA sequences or cDNA sequences encoding proteins can be isolated based on the amino acid sequence of proteins or certain protein properties. Many methods of sequence isolation are known in the art of molecular biology. See particularly Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Plainview, New York), herein incorporated by reference.

To produce an antithrombin polypeptide by recombinant DNA technology, a gene encoding a polypeptide of the invention is prepared. The DNA coding sequence typically does not contain introns. The DNA sequence is isolated and purified, the gene is inserted in an expression vector able to drive expression and production of the recombinant product. The DNA sequence may be a cDNA sequence. The DNA sequence may be a synthetic DNA sequence. The synthetic gene is typically prepared by chemically synthesizing oligonucleotides which, in total, correspond to the desired gene. The synthesized oligonucleotides are then assembled to obtain the gene.

If desired, the gene sequence may be modified by site-directed mutagenesis to introduce one or more coding changes. Typically, a gene is constructed with restriction sites at each end to facilitate its subsequent manipulation.

A DNA sequence may be provided which further encodes a leader peptide. The leader peptide is capable of directing secretion of the polypeptide from cells in which the polypeptide is to be expressed. The sequence encoding the leader peptide is typically fused to the 5'-end of the DNA sequence encoding the polypeptide.

Leader sequences are known in the art and include the OmpA leader peptide, the leader peptide of vesicular stomatitis virus G protein (VSV G protein). The OmpA leader is useful when expression is in a bacterial host, such as *E. coli* while the VSVG protein is useful when expression is in insect cells.

The DNA sequence may be provided with a cleavable site to release the polypeptide of the invention. A DNA sequence may be used which encodes a carrier polypeptide sequence fused via a cleavable linkage to the end-terminus of a polypeptide of the invention. The cleavable linkage may be one cleavable by cyanogen bromide.

For expression of the polypeptides, an expression vector is constructed which comprises a DNA sequence encoding the polypeptide which is capable of expressing the polypeptide in a suitable host. Appropriate transcriptional and translational control elements are provided, including a promoter for the DNA sequence, a transcriptional termination site, and translation start and stop codons. The DNA sequence is provided in the correct frame such as to enable expression of the polypeptide to occur in a host compatible with the vector.

The expression vector typically comprises an origin of replication and, if desired, a selectable marker gene such as antibiotic resistance. The expression vector may be a plasmid, a virus, particularly a baculovirus, and the like.

Once the nucleotide sequences encoding the antithrombin proteins of the invention have been isolated, they can be manipulated and used to express the protein in a variety of hosts including other organisms, including microorganisms.

Once the nucleotide sequence is identified and known, those skilled in the art can produce large quantities of the protein for therapeutic use. Accordingly, recombinant protein and methods for producing the recombinant protein are encompassed by the present invention. In this manner, the nucleotide sequence encoding the antithrombin protein can be utilized in vectors for expression in various types of host cells, including both prokaryotes and eukaryotes, to produce large quantities of the protein, or active analogues, or fragments thereof, and other constructs having antithrombin activity.

Generally, methods for the expression of recombinant DNA are known in the art. See, for example, Sambrook *et al.*, *Molecular Cloning*, Cold Spring Harbor Laboratory (1989). Additionally, host cells and expression vectors, such as the baculovirus expression vector may be employed in carrying out the present invention, as described in U.S. Patent Nos. 4,745,051 and 4,879,236. In general, a baculovirus expression vector comprises a baculovirus genome containing the gene to be expressed inserted into the polyhedron gene at a position ranging from the polyhedron transcriptional start signal to the ATG start site and under the transcriptional control of a baculovirus polyhedron promoter.

A broad variety of suitable prokaryotic and microbial vectors are available. Likewise, the promoters and other regulatory agents used in expression of foreign proteins are available in the art. Promoters commonly used in recombinant microbial expression vectors are known in the art and include the beta-lactamase (penicillinase) and lactose promoter systems (Chang *et al.* (1978) *Nature*, 275:615 and Goeddel *et al.* (1979) *Nature*, 281:544); A tryptophan (TRP) promoter system (Goeddel *et al.* (1980) *Nucleic Acids Res.*, 8:4057 and the EPO Application Publication No. 36,776); and the Tac promoter (DeBoer *et al.* (1983) *Proc. Natl. Acad. Sci. USA*, 80:21). While these are commonly used, other microbial promoters are available. Details concerning nucleotide sequences of many have been published, enabling a skilled worker to operably ligate them to DNA encoding the protein in plasmid or viral vectors. See, for example, Siedenlist *et al.* (1980) *Cell*, 20:269.

Eukaryotic microbes such as yeast may be transformed with suitable protein-encoding vectors. See, e.g., United States Patent No. 4,745,057.

Saccharomyces cerevisiae is the most commonly used among lower eukaryotic host microorganisms, although a number of other strains are commonly available. Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding the desired protein, sequences for polyadenylation and transcription termination, and a selection gene. An exemplary plasmid is YRp7, (Stinchcomb *et al.* (1979) *Nature*, 282:9; Kingsman *et al.* (1979) *Gene*, 7:141; Tschemper *et al.* (1980) *Gene*, 10:157). This

to complex or absorb the compositions. The controlled delivery may be exercised by selecting appropriate macromolecules (for example, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinylacetate, methylcellulose, carbosymethylcellulose, or protamine sulfate). The rate of drug release may also be controlled by altering the concentration of such macromolecules.

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Another possible method for controlling the duration of action comprises incorporating the therapeutic agents into particles of a polymeric substance such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, it is possible to entrap the therapeutic agents in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethyl cellulose or gelatin-microcapsules or poly(methylmethacrylate) microcapsules, respectively, or in a colloid drug delivery system, for example, liposomes, albumin, microspheres, microemulsions, nanoparticles, nanocapsules, or in macroemulsions. Such teachings are disclosed in *Remington's Pharmaceutical Sciences* (1980).

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In more specific embodiments, a polypeptide of the invention may be converted into a pharmaceutically acceptable salt. It may be converted into an acid additional salt with an organic or inorganic acid. Suitable acids include acetic, succinic and hydrochloric acid. Alternatively, the peptide may be converted into a carboxylic acid salt such as the ammonium salt or an alkali metal salt such as the sodium or potassium salt.

A polypeptide or pharmaceutically acceptable salt thereof may be used in a pharmaceutical composition, together with a pharmaceutically acceptable carrier or excipient therefor. Such a formulation is typically for intravenous administration (in which case the carrier is generally sterile saline or water of acceptable purity). A polypeptide can therefore be used for the therapy and prophylaxis of thromboses and thromboembolisms in a human or other mammal, including the prophylaxis of post-operative thromboses, for acute shock therapy (for example for septic or polytraumatic shock), for the therapy of consumption coagulopathics, in hemodialyses, haemoseparations and in extracorporeal blood circulation. In one

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embodiment of the invention, the polypeptide or salt thereof can be coadministered with a plasminogen activator, such as tissue plasminogen activator.

The dosage depends especially on the specific form of administration and on the purpose of the therapy or prophylaxis. The size of the individual doses and the administration regime can best be determined by way of an individual judgment of the particular case of illness; the methods of determining relevant blood factors required for this purpose are familiar to the person skilled in the art. Normally, in the case of an injection the therapeutically effective amount of the compounds according to the invention is in a dosage range of from approximately from .005 or 5 .01 to approximately .05 or .1 mg/kg body weight, preferably from approximately 10 0.01 to approximately 0.05 mg/kg body weight.

The administration is effected by intravenous, intramuscular or subcutaneous injection. Accordingly, pharmaceutical compositions for parenteral administration in single dose form contain per dose, depending on the mode of administration, from approximately 0.4 to approximately 7.5 mg of the compound according to the invention. In addition to the active ingredient these pharmaceutical compositions usually also contain a buffer, for example a phosphate buffer, which is intended to keep the pH value between approximately 3.5 and 7, and also sodium chloride, mannitol or sorbitol for adjusting the isotonicity. The preparations may be freeze-dried or dissolved. An antibacterially active preservative may be included, for example from 0.2 to 0.3% 4-hydroxybenzoic acid methyl ester or ethyl ester.

A composition for topical application can be in the form of an aqueous solution, lotion or gel, an oily solution or suspension or a fat-containing or, especially, emulsified ointment. A composition in the form of an aqueous solution is obtained, for example, by dissolving the active ingredients according to the invention, or a therapeutically acceptable salt thereof, in an aqueous buffer solution of from e.g., pH 4 to pH 6.5 and, if desired, adding a further active ingredient, for example an anti-inflammatory agent, and/or a polymeric binder, for example polyvinylpyrrolidone, and/or a preservative. The concentration of active ingredients is from approximately 0.1 to approximately 1.5 mg, preferably from 0.25 to 1.0 20 mg, in 10 ml of a solution or 10 g of a gel.

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An oily form of administration for topical application is obtained, for example, by suspending the active ingredient according to the invention, or a therapeutically acceptable salt thereof, in an oil, optionally with the addition of swelling agents, such as aluminum stearate, and/or surfactants (tensides) having an HLB value ("hydrophilic-lipophilic balance") of below 10, such as fatty acid monomers of polyhydric alcohols, for example glycerin monostearate, sorbitan monolaurate, sorbitan monostearate or sorbitan monooleate. A fat-containing ointment is obtained, for example, by suspending the active ingredient according to the invention, or a salt thereof, in a spreadable fatty base, optionally with the addition of a tenside having an HLB value of below 10. An emulsified ointment is obtained by triturating an aqueous solution of the active ingredient according to the invention, or a salt thereof, in a soft, spreadable fatty base with the addition of a tenside having an HLB value of below 10. All these forms for topical application can also contain preservatives. The concentration of active ingredient is from approximately 0.1 to approximately 1.5 mg, preferably from 0.25 to 1.0 mg, in approximately 10 g of base.

In addition to the compositions described above and pharmaceutical compositions analogous thereto that are intended for direct medicinal use in the body of a human or a mammal, the present invention relates also to pharmaceutical compositions and preparations for medicinal use outside the living body of humans or mammals. Such compositions and preparations are used especially as anticoagulant additives to blood that is being subjected to circulation or treatment outside the body (for example haemoseparation). Such preparations, such as stock solutions or alternatively preparations in single dose form, are similar in composition to the injection preparations described above; however, the amount of concentration of active ingredient is advantageously based on the volume of blood to be treated or, more precisely, on its thrombin content. Depending on the specific purpose, the suitable dose is from approximately 0.01 to approximately 1.0 mg of the active ingredient/liter of blood, although the upper limit may still be exceeded without risk as the agent is harmless even in relatively high amounts.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

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Collection of salivary glands

Simulium vittatum females were taken from a continuous laboratory culture maintained at the University of Arizona (Bernardo et al., (1986), *Ann. ent. Soc. Am* 79:610-621). Salivary glands were removed surgically from females maintained on water *ad libitum* for 48 h to enhance optimum protein secretion prior to dissection. (Cupp et al. (1993), *J. Insect Physiol.* 39:817-821). Glands were removed on a clean glass slide in chilled 0.15 M NaCl (saline) and transferred to an Eppendorf tube containing 50 μ l of chilled saline on ice. Up to 250 glands were accumulated per vial and stored at -70°C until needed.

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Physical characterization of S. vittatum anti-thrombin

Crude salivary gland lysate was used to determine heat stability (56°C for 1 h), alcohol precipitation (90% ethanol), trypsinization for 30 min and stability to 60% acetonitrile and 0.1% trifluoracetic acid (TFA) (1 h). Thrombin inhibition assays were conducted as described by Abebe et al. (1994), *J. med. Ent.* 31:908-911.

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Anticoagulant purification

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One thousand eight hundred and fifty glands were lysed by four cycles of rapid freeze-thawing (between liquid N₂ and 37°C). The lysate was spun at 10,000 g for 15 min at 4°C and the supernatant fluid was removed directly from the Eppendorf tube with the aid of a tuberculin syringe fitted with a plastic automatic pipette tip. One hundred microliters of saline was drawn into the syringe with lysate and gently mixed. The pipette tip was replaced with a 0.2 μ m pore size Acrodisc 13 filter (Gelman Sciences, Ann Arbor, MI) and the salivary suspension was directly applied to a 10 kDa molecular weight cut-off Centricon filter (Amicon, Beverly, MA). Two hundred microliters of saline were passed through the filter

membrane twice and added to the salivary proteins which were concentrated by centrifugation using a JA-20 rotor at 4100 rpm in a Beckman JA 20-21 induction drive centrifuge (Beckman, Fullerton, CA) for 1.5 h at 4°C. The retentate contained most of the activity and was used as starting material for purification of the anticoagulant components using an LDC Analytical (thermo Separation Products, Fremont, CA) high performance liquid chromatography (HPLC) system.

The anticoagulant was isolated by a two-step purification procedure using a reversed phase C-8 macrosphere column (Alltech, Dearfield, IL). In both steps the mobile phases were 15% acetonitrile/water (A) and 50% acetonitrile/water (B), each containing 0.1% TFA. The first purification step followed the protocol of 0-10 min at 100% A, 10-70 min with a gradient of 0-100% B, then 70-80 min at 100% B. The protocol for the second purification step was 0.10 min at 100% A, followed by gradients of 10-24 min = 0-39% B, 24-52 min = 39-49% B, then 52-70 min at 49% B. Fractions of 1 ml were collected in Eppendorf Tubes with the aid of a Gilson FC-100K fractionator (Gilson, Middleton, WI). Ten microliters of a 1% bovine serum albumin (BSA) solution were added to a 50 μ l aliquot of each fraction before drying in a Juan RC1010 centrifugal vacuum concentrator (Juna, Los Gatos, CA) and stored at -70°C until needed.

Detection and isolation of fractions anti-thrombin activities

Anti-thrombin activities for all fractions were measured as described by Abebe et al. (1994), *J. med. Ent.* 31:908-911, except that thrombin was reduced to 3.5 ng. To rule out possible cross activity against factor Xa and to determine where the fraction with anti-factor Xa activity eluted in relation to the anti-thrombin, the enzyme inhibition assay for Xa was conducted using the procedure described above with the same modification. Tests were done in duplicate for each fraction. For further purification, the fraction with anti-thrombin activity was rechromatographed under the same conditions with a modified gradient. Anti-thrombin activity was tested for each of the fractions as outlined above. Inhibition was expressed as a percentage of the control which contained only BSA. Anticoagulant activity of the anti-thrombin fraction was determined with the recalcification time test.

Determination of molecular weight, amino acid composition and N-terminal sequencing

The determination of the molecular weight by matrix assisted laser desorption mass spectrometry (MALD-MS) (Hillenkamp et al. (1991), *Analyt. Chem.* 63:1193-1202) and amino acid composition and N-terminal sequencing was made at the Harvard Microchemistry Facilities (Cambridge, MA). Version 7 of the GCG program (Genetics Computer Group, Inc., Madison, WI) was used to search for N-terminal sequence homology.

PCR Production of cDNA for Coding Region

Knowledge of the N-terminal protein amino-acid sequence was used to construct degenerate, forward primers which were used along with a polymerase chain reaction (PCR) technique known as 3' RACE (random amplification of cDNA ends), to amplify specific target DNA. The DNA pool was generated by reverse-transcription of *S. vittatum* salivary gland mRNA. The oligo-dT template (16T) used to generate the DNA pool had 13 extra bases attached to the 5' end which resulted in a known "tag" being added to each cDNA. A PCR that included the tagged cDNA, the anti-thrombin specific degenerate primers and the tagged oligo-dT primer generated a product of approximately 700 base pairs. See, SEQ ID NO:

The PCR product was gel purified, quantified and cloned using commercially-available Invitrogen TA Cloning Vector. The cDNA was sequenced by methods available in the art and the sequence is provided in SEQ ID NO: 1.

Translation of the cDNA code produced the putative amino acid sequence for the mature protein. The calculated molecular weight of the putative protein (11,302 Daltons) is slightly less than the molecular weight of the pure, active protein isolated from *S. vittatum* SGE and determined by mass spectroscopy ($11,334 \pm 28$ Daltons). This may indicated that some post-translational modification to simulidin occurs during protein translation.

The presence of more than 200 bases beyond the stop codon at the 3' end of the DNA suggests that the message for this protein is highly regulated by *S. vittatum*.

salivary gland cells *in vivo*. Analysis of the putative amino acid structure indicates that the mature protein contains 3 disulfide bonds which may be important to function. In addition, the C-terminus is highly acidic, which is a feature similar to the well-studied anti-thrombin protein, hirudin.

5 All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

10 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

15 Many modifications and other embodiments of the invention will come to mind in one skilled in the art to which this invention pertains having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed. Although specific terms are employed, they are used in a generic and descriptive sense only and not for purposes of limitation, and that 20 modifications and embodiments are intended to be included within the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Cupp, Mary S.
Cupp, Eddie W.

(ii) TITLE OF INVENTION: ANTITHROMBIN PROTEIN AND DNA SEQUENCES

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: W. Murray Spruill (Alston & Bird, LLP)
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(D) STATE: NC
(E) COUNTRY: USA
(F) ZIP: 27622

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Spruill, W. Murray
(B) REGISTRATION NUMBER: 32,943
(C) REFERENCE/DOCKET NUMBER: 5721-5

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 919 420 2202
(B) TELEFAX: 919 881 3175

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 532 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Simulium vittatum

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 1..294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAG GTG GCG AAT TTG CAG GAC CAT CGA GCT GTT GAA TTT GTG TGC GAG Glu Val Ala Asn Leu Gln Asp His Arg Ala Val Glu Phe Val Cys Glu	15	48
1 5 10 15		
 AAG GAT ACT GAA AAC CAG CAT GGT TCC GAT TGC CTG CTT TCT TGT GAC Lys Asp Thr Glu Asn Gln His Gly Ser Asp Cys Leu Leu Ser Cys Asp	30	96
20 25 30		
 GTG ATG TTC TGG GAT ACC AAA AAC GAG AAC AAC AAG GAA TAT GAA GAC Val Met Phe Trp Asp Thr Lys Asn Glu Asn Asn Lys Glu Tyr Glu Asp	45	144
35 40 45		
 AGA TAC AAT TTG TGC AAA CAT TCA GCC GCT TCC GAA GAG AAC ATT TGT Arg Tyr Asn Leu Cys Lys His Ser Ala Ala Ser Glu Glu Asn Ile Cys	60	192
50 55 60		
 GAT CGC AAT GAA GAA TTG AGA GCC TGT TTC TTG CAT GAT TCG TCA TAC Asp Arg Asn Glu Glu Leu Arg Ala Cys Phe Leu His Asp Ser Ser Tyr	80	240
65 70 75 80		
 GAA GAG ACT TCG GAC GAA TAT GAA ATA ACC TAC AGC ATG GAT TCC CTG Glu Glu Thr Ser Asp Glu Tyr Glu Ile Thr Tyr Ser Met Asp Ser Leu	95	288
85 90 95		
 TGA TGA TCAAAACATTG GTAATAGTTC AATTGATCGA AATATGCAGA AACCGTCCAC	344	
* *		
 GGTAGTGTAA TTATAACCCA TGTTGTTCGC ATTGTACTCT AATTCTACTC CGTCATATA	404	
 TGGCTGATGA GTGCCATCCA GCCAATGTGA AACAGGAGTA TAAAAAGCAC AATGTGGGTG	464	
 ACAGTCCCCAT TCACACACAATA TGCAAATAAA ATAATGGAAA TGACCCCCAAA AAAAAAAAAA	524	
 AAAAAAAAA	532	

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 98 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu	Val	Ala	Asn	Leu	Gln	Asp	His	Arg	Ala	Val	Glu	Phe	Val	Cys	Glu
1				5					10					15	
Lys	Asp	Thr	Glu	Asn	Gln	His	Gly	Ser	Asp	Cys	Leu	Leu	Ser	Cys	Asp
				20				25					30		

Val Met Phe Trp Asp Thr Lys Asn Asn Asn Lys Glu Tyr Glu Asp
35 40 45

Arg Tyr Asn Leu Cys Lys His Ser Ala Ala Ser Glu Glu Asn Ile Cys
50 55 60

Asp Arg Asn Glu Glu Leu Arg Ala Cys Phe Leu His Asp Ser Ser Tyr
65 70 75 80

Glu Glu Thr Ser Asp Glu Tyr Glu Ile Thr Tyr Ser Met Asp Ser Leu
85 90 95

* *

THAT WHICH IS CLAIMED:

- 1 1. A substantially purified protein having anti-thrombin activity,
2 wherein said protein is isolated from the salivary glands of a species of the order
3 Nematocera.
- 1 2. The protein of claim 1 wherein said protein comprises the amino acid
2 sequence given in SEQ ID NO: 2.
- 1 3. The protein of claim 2, wherein said protein is isolated from the
2 salivary glands of a species of *simulium*.
- 1 4. The protein of claim 3, wherein said species is selected from the
2 group consisting of *S. vittatum*, *S. metallicum*, *S. bivittatum*, *S. argus*, and *S.*
3 *ochraceum*.
- 1 5. The protein of claim 4, wherein said species is *S. vittatum*.
- 1 6. The protein of claim 1, wherein said protein is produced by
2 recombinant methods.
- 1 7. An isolated nucleotide sequence which encodes a protein having anti-
2 thrombin activity, wherein said protein is isolated from the salivary glands of a
3 species of the order Nematocera.
- 1 8. The nucleotide sequence of claim 7, wherein said protein comprises
2 the amino acid sequence set forth in SEQ ID NO: 2.
- 1 9. The nucleotide sequence of claim 8, wherein said sequence comprises
2 the DNA sequence set forth in SEQ ID NO: 1.

1 10. A nucleotide sequence that hybridizes to the sequence of claim 8
2 under stringent conditions.

1 11. A vector comprising the nucleotide sequence of claim 8.

1 12. A host cell comprising the vector of claim 11.

1 13. A vector comprising the nucleotide sequence of claim 9.

1 14. A host cell comprising the vector of claim 13.

1 15. A method for producing a protein having anti-thrombin activity, said
2 method comprising:

3 culturing a procaryotic or eucaryotic cell that is transformed with a
4 nucleotide sequence encoding the protein of claim 2 under conditions such that said
5 protein is produced; and,

6 isolating said protein.

1 16. A method for treating venous thrombosis in a mammal, said method
2 comprising administering a therapeutically effective amount of an anti-thrombin
3 protein, wherein said protein comprises the amino acid sequence set forth in SEQ ID
4 NO: 2 to said mammal.

1 17. The method of claim 16, wherein said protein is produced by
2 recombinant methods.

TITLE OF THE INVENTION
ABSTRACT OF THE DISCLOSURE

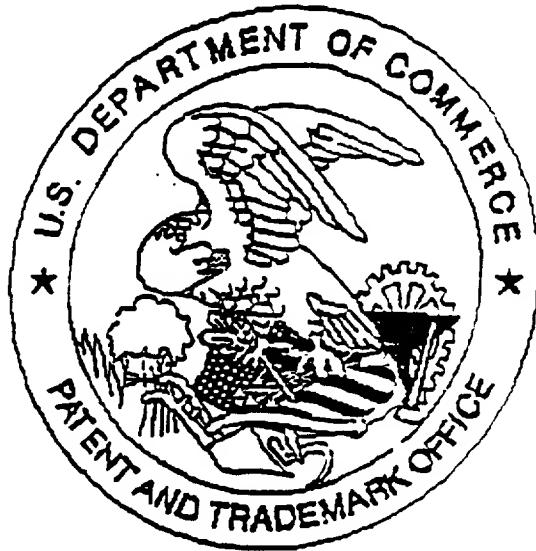
5

The invention is drawn to anti-thrombin proteins from the salivary glands of the species, *Simulium*. Methods for recombinant production of the protein as well as biomedical uses are provided.

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